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Noxious Effect of Trace Metals on Probiotic "*Lactobacillus rhamnosus*"

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ABSTRACT:
The sensile of probiotic strain to trace metals was examined by performing Minimum inhibitory concentration assay (MIC). In present study, *Lactobacillus rhamnosus* is one of best educed probiotic, which shows its therapeutic competency and impregnability over a wide range of trace elements, but current study reveals the lenity to two metals *viz.*, Cu^{2+} and Zn^{2+} cultured at 37°C for 10 hr in MRS broth without shaking .bacterial growth was measured for every one hour using spectrophotometer. The sensile nature of *L.rhamnosus* was correlated with that of cell grown in the absence of trace metals broth which acts as control. Our observation confessed the amendment in the growth profiles pattern of *L.rhamnosus* exposed to trace metals. Conclusively our results invincibly broach that trace metals are noxious at higher concentration which may be able to interact with the metabolism of Probiotic *L.rhamnosus* strain.

Key words: *Lactobacillus rhamnosus,* Trace metals, Noxious

INTRODUCTION

Lactobacillus contains a very large number of species, isolated mainly from humans, animals, plants and foods; the genus shows large phenotypic, biochemical and physiological variability [1, 2]. *Lactobacilli* are used as starters to manufacture of cheeses, yoghurt, sourdough breads, silage, table olives, sauerkraut, fermented fish and sausages, and have been proposed as natural bio-preservatives in non-fermented vegetables. Several health benefits (probiotic) are also reported with regard to *Lactobacilli* strains that colonize the gastrointestinal tract. These include stimulation of immunoglobulin production, induction of interferon expression in macrophages, acidification of the local environment, hypocholesteraemic effects, binding of mutagenic compounds, production of bacteriocins, and prevention of the adhesion of pathogenic bacteria to the epithelial cell [3&4]. In future, *Lactobacilli* will probably include the construction of strains capable of producing essential nutrients and enzymes or displaying epitomes' as components in oral vaccines. The general utility of *Lactobacillus* species is related to their GRAS (Generally Recognized as Safe) status. Their large-scale use will be dependent on the availability of cost-effective methods for the production and delivery of viable cultures [5].

Lactobacilli are exposed to various environmental stresses such as metal stress extremes in temperature, pH, osmotic pressure, oxygen, high pressure and starvation which may affect the physiological status and properties of the cells. It is essential to know not only which conditions are favorable or detrimental for the life of *Lactobacilli* but also which mechanisms permit their survival and metabolic activities under stress conditions. The field of environmental stress responses is vast and the related study of the proteomics of lactic acid bacteria(LAB) has generated increasing interest in recent years [6&7].

Lactobacillus rhamnosus is originally considered to be a subspecies of *L. casei*, but later genetic research found it to be a species of its own. Some strains of *L. rhamnosus* are being used as probiotics. The species is sometimes used in yogurt and other dairy products. Literature reveals that *L. rhamnosus* considered as a beneficial organism, and noticed *to* be pathogenic in certain circumstances [8].

Copper is used by cells in small quantities in cellular enzymes (e.g., cytochrome c oxidase). However, because copper is so widely used in mining, industry, and agriculture, high levels of copper may exist in environment. As such, bacteria have evolved several types of mechanisms to resist toxicity due to high copper concentrations. With respect to the prevalence of copper resistance in the environment, bacteria from a water distribution system experiencing copper corrosion, and 62% were found to be copper resistant[9]. Of these resistant bacteria 49% had *cop* or *cop*-like gene systems, including both compartmentalization and efflux systems [10]. In *E. coli*, resistance to copper is based on an efflux mechanism by which copper is removed from the cell. The efflux proteins are expressed by plasmid-bound *pco* genes, which are in turn are dependent on the expression of chromosomal *cut* genes [10]. Two *cut* genes (*cutC* and *cutF*) were identified and were shown to encode a copper binding protein and an outer membrane lipoprotein. The most bacterial species in the environment have acquired at least one of the afore mentioned copper management systems, and that the evolution of copper resistance may have

come about through the modification of copper uptake genes found on chromosomes [11].

Zinc is another essential trace element. It is not biologically redox reactive and is thus not used in respiration. It is, however, important in forming complexes and as a component in cellular enzymes[12].Bacterial cells accumulate zinc by a fast, unspecific uptake mechanism and it is normally found in higher concentrations (but is less toxic) than other heavy metals [12]. Uptake of zinc ions is generally coupled to that of magnesium, and the two ions may be transported by similar mechanisms in bacteria [13] Two general efflux mechanisms are responsible for bacterial resistance to zinc. One is a P-type ATPase efflux1 system that transports zinc ions across the cytoplasmic membrane by energy from ATP hydrolysis. A chromosomal gene, *zntA*, was isolated from *E. coli* K-12 and was found to be responsible for the ATPase that transports zinc and other cations across cell membranes [14]. The other mechanism involved in zinc efflux is an RND-driven2 transporter system that transports zinc across the cell wall (not just the membrane) of gram-negative bacteria and is powered by a proton gradient and not ATP [12].

When copper and Zinc are taken in excess, bacterial cell become sensitive and it may disturb the bacterial metabolic systems. When copper and Zinc stressed probiotic bacteria enters in to our body. It may cause metabolic changes and may also became pathogenic. In dilution tests, microorganisms are tested for their abilityto produce visible growth on a series of agar plates (agar dilution) or in microplate wells of broth (broth microdilution) containing dilutions of the antimicrobial agent. The lowest concentration of an antimicrobial agent, that will inhibit the visible growth of microorganisms known as the MIC. In the present study, we designed experiments and performed the Minimum inhibitory concentration by macrodilution method (Anne Spain 2003) which lead to estimates the growth curves of *Lactobacillus rhamnosus* (mtcc 1408) against Copper sulphate and Zinc chloride.

MATERIALS AND METHODS

Preparation of inoculum: The density of *L.rhamnosus* inoculum to give 10^4 colony-forming units (CFU) per spot on the agar was standardized. The inoculum was prepared by emulsifying overnight colonies diluting a broth culture. A 0.5 McFarland standard was used for visual comparison to adjust the suspension to a density equivalent to approximately 10^8 CFU/mL. Alternatively, inoculum can be adjusted photo metrically [16].

Determination of minimum inhibitory concentration for Copper Sulphate and Zinc chloride: The MIC of the two metals, i.e., Cu and Zn was determined by macro dilution method [17].

For determination of minimum inhibitory concentration of Copper sulphate and Zinc chloride, ten 50 ml conical flasks were taken for each metal. For each flask, 10 ml of MRS broth was added. The range of metal salts concentration tested will depend on the organisms and metal salts being tested. To the 5 flasks, 0 mM, 20 mM, 40 mM, 60 mM, 80 mM concentrations of copper sulfate and Zinc chloride was added respectively. The remaining 5 flasks kept for control. In these flasks respective concentration of metals added without adding culture. To the test samples 10 µL of inoculum (O.D 0.5 McFarland standard) was added to each different concentrations of copper sulfate and Zinc chloride. Then the flasks were incubated for 24hrs at 37^0C under aerobic conditions. After incubation, the optical densities for each concentration were recorded at 520nm. The lowest concentration of Copper sulphate and Zinc chloride, that completely prevent the growth of *Lactobacillus rhamnosus* were analyzed.

Evaluation of growth curves:

Growth curves of the bacterial strains were determined by the amount of biomass produced in relation to time[15]. Parent strains were fully grown in MRS media at 37^0C for 18 hr, and approximately 5.2×10^7 CFU of each strain were inoculated into each of a series of tubes containing 9.9 ml of MRS broth with copper and zinc concentrations consisting of doubling dilutions below and above the MIC and incubated at 37^0C for 24 hrs. After incubation, aliquots from the tube nearest the MIC were used following a 1:100 dilution to inoculate a second set of tubes containing MRS broth with Copper sulphate and Zinc chloride and incubated overnight at 37^0C. After incubation, the bacteria were transferred again and 10-12 serial transfers were carried out. Likewise each strain was then cultivated in doubling concentration.

An approximately equal number of cells ($2\times10^{6)}$ of normal parental strains and the respective copper influenced and zinc influenced strains of *Lactobacillus rhamnosus* were grown in MRS media

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at 37^{0}C. Bacterial growth was measured at different time intervals using spectrophotometer at 570nm.

RESULTS:

The minimum inhibitory concentration of the $CuSO_4$ was tested against *L. rhamnosus*

Table:1 The minimum inhibitory concentration of the $CuSO_4$ was tested against *L. rhamnosus.*

	O.D values		
Concentration of metal	Control (C)	Test (T)	Difference (T-C)
0 mM	0.12	-	-
20 mM	0.015	0.095	0.080
40mM	0.071	0.13	0.059
60mM	0.12	0.15	0.03
80mM	0.16	0.18	0.02
100mM	0.19	0.20	0.01

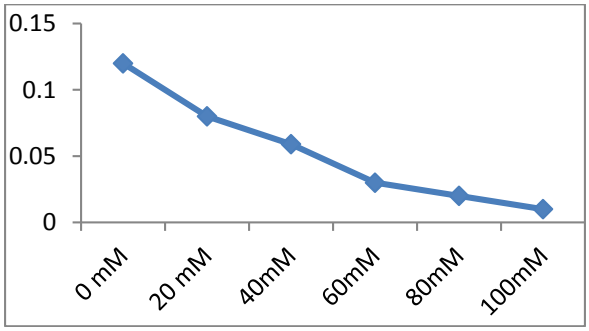

Fig 1:Line graph represents the Inhibition of *L.rhamnosus* by the trace metal Copper sulphate. At 40 mM concentration of Copper sulphate, *L. rhamnosus* showed 50% inhibition.

2. Evaluation of Growth curves of Copper Sulphate stressed *L.rhamnosus.*

Table:2 Growth curves of 40 mM $CuSO_4$ stressed *L.rhamnosus*

	O.D Values	
Time interval	Control	Test
0 hr	0.00	0.00
1 hr	0.030	0.012
2 hr	0.064	0.034
3 hr	0.092	0.058
4 hr	0.13	0.084
5 hr	0.17	0.11
6 hr	0.23	0.16
7 hr	0.27	0.19
8 hr	0.27	0.19
9 hr	0.22	0.17

1. Determination of minimum inhibitory concentration for copper Sulphate:

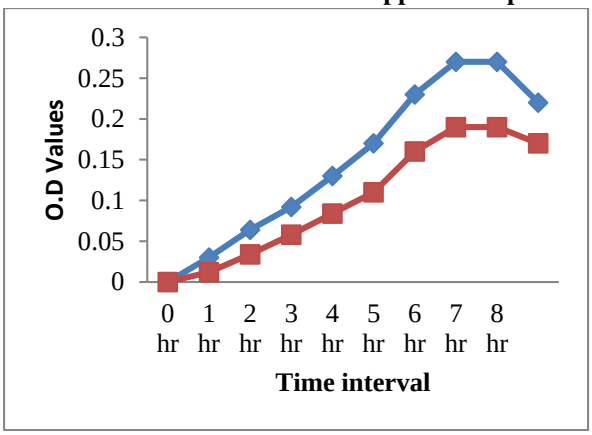

Fig 2: Log growth of $CuSO_4$*L. rhamnosus* mtcc 1408 over hours 1hr to 8hr. Microbial growth was measured every 45 min.

Table:3 The minimum inhibitory concentration of the $ZnCl_2$ tested against *L.rhamnosus.*

	O.D values		
Concentration of metal	Control (C)	Test (T)	Difference (T-C)
0 mM	0.035	-	-
20 mM	0.016	0.045	0.029
40mM	0.017	0.042	0.025
60mM	0.019	0.038	0.019
80mM	0.023	0.036	0.013
100mM	0.028	0.032	0.004

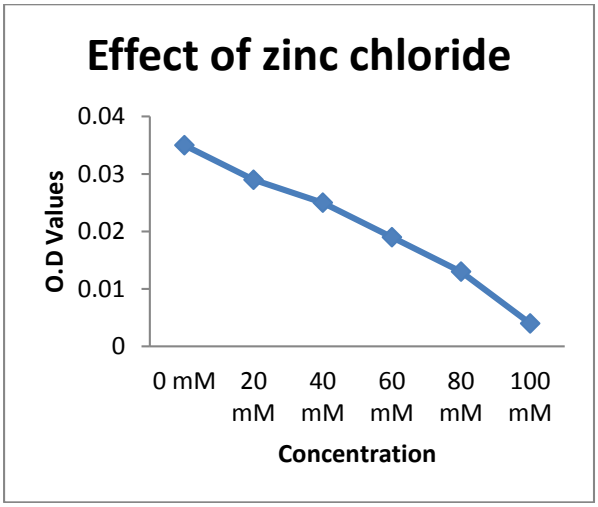

Fig 3:Line graph represents the inhibition of *L .rhamnosus* by the trace metal Zinc chloride. At 40 mM concentration of Zinc chloride, *L. rhamnosus* showed 50% inhibition.

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Table:4 Growth curves of 40 mM $ZnCl_2$stressed *L. rhamnosus*

Time interval	O.D Values	
	Control	Test
0 hr	0.00	0.00
1 hr	0.023	0.011
2 hr	0.048	0.025
3 hr	0.069	0.041
4 hr	0.09	0.06
5 hr	0.14	0.08
6 hr	0.19	0.10
7 hr	0.19	0.11
8 hr	0.17	0.09
9 hr	0.15	0.06

Graph:4
Growth curves of 40 mM $ZnCl_2$ stressed *L.rhamnosus*

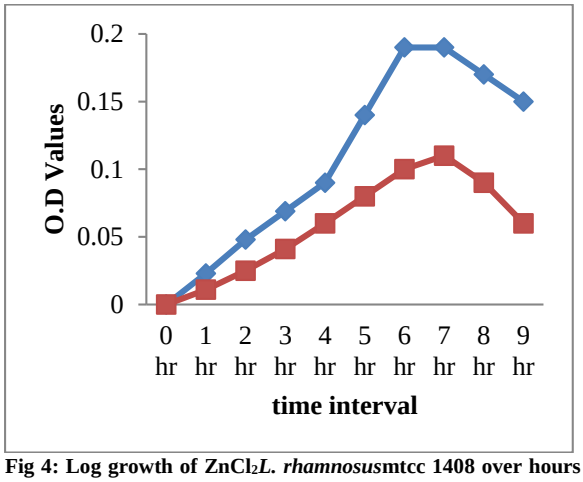

Fig 4: Log growth of $ZnCl_2$*L. rhamnosus*mtcc 1408 over hours 1hr to 8hr. Microbial growth was measured every 45 min.

DISCUSSION:

Initially, $CuSO_4$ and $ZnCl_2$ was used in concentrations of 20mM, 40 mM, 60mM, 80 mM and 100 mM for*L.rhamnosus.* However, minimum inhibition was observed to be probable at 40 mMfor both $CuSO_4$ and $ZnCl_2$ for *L.rhamnosus*. Subsequent tests with higher concentrations confirmed that,at 100mM concentration of both $CuSO_4$ and $ZnCl_2$ shows least inhibition for *L. rhamnosus*, with remarkable growth decrease in the range of 0.02 and 0.004 respectively. **(Tables 1&2 and Graphs 1&2).**Further study was conducted by considering the minimum inhibitory concentration i.e., 40 mM at which 50 % inhibition was seen. Growth rate of test organism under $CuSO_4$ and $ZnCl_2$ metal stress were tabulated at different time intervals, graphical representation denotes that test organism's growth increases with increase in time period to certain extent and then decreases **(Table 3 & 4 and (Graphs 3& 4**). Advance of this study includes PAGE, 2D PAGE and MALDI-TOF.

CONCLUSION:

Although some trace metals are important and essential elements, at high concentrations most of the trace metals can be proved as toxic to microbes. Further, Proteomic studies subsequent microarray analysis and other molecular biological approaches need to be performed to assign to document toxicity of trace metals on probiotic *L. rhamnosus*. The understanding of bacterial metal toxicity system has been useful for both environmental sciences and medicine.

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